Maldonado, M. E., Oh, K., & Frey, P. A. (1972) *J. Biol. Chem. 247*, 2711-2716.
Reed, L. J. (1966) in *Comprehensive Biochemistry* (Florkin, M., & Stotz, E., Eds.) Vol. 14, p 103, Elsevier, New York.
Reed, L. J., & Willms, C. R. (1966) *Methods Enzymol. 13*, 55.
Rose, I. A., Grunberg-Manago, M., Korey, S. R., & Ochoa, S. (1954) *J. Biol. Chem. 211*, 737-756.
Schwartz, E. R., & Reed, L. J. (1970) *J. Biol. Chem. 245*, 183-187.
Siegal, J. M., Montgomery, B. A., & Bock, R. M. (1959) *Arch. Biochem. Biophys. 82*, 288-299.
Speckhard, D. C. (1974) Ph.D. Dissertation, The Ohio State University, Columbus, OH.
Stephens, P. E., Darlson, M. G., Lewis, H. M., & Guest, J. R. (1983) *Eur. J. Biochem. 133*, 155-162.
Sümegi, B., & Alkonyi, I. (1983) *Arch. Biochem. Biophys. 223*, 417-424.
Vogel, O., & Henning, U. (1971) *Eur. J. Biochem. 18*, 103-115.
Wostman, B. S., & Knight, P. L. (1960) *Experientia 16*, 500-501.

Alternative Proton Donors/Acceptors in the Catalytic Mechanism of the Glutathione Reductase of *Escherichia coli*: The Role of Histidine-439 and Tyrosine-99†

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ABSTRACT: The cloned *Escherichia coli gor* gene encoding the flavoprotein glutathione reductase was placed under the control of the *tac* promoter in the plasmid pKK223-3, allowing expression of glutathione reductase at levels approximately 40 000 times those of untransformed cells. This greatly facilitated purification of the enzyme. By directed mutagenesis of the *gor* gene, His-439 was changed to glutamine (H439Q) and alanine (H439A). The tyrosine residue at position 99 was changed to phenylalanine (Y99F), and in another experiment, the H439Q and Y99F mutations were united to form the double mutant Y99FH439Q. His-439 is thought to act in the catalytic mechanism as a proton donor/acceptor in the glutathione-binding pocket. The H439Q and H439A mutants retain ~1% and ~0.3%, respectively, of the catalytic activity of the wild-type enzyme. This reinforces our previous finding [Berry et al. (1989) *Biochemistry 28*, 1264-1269] that direct protonation and deprotonation of the histidine residue are not essential for the reaction to occur. The retention of catalytic activity by the H439A mutant demonstrates further that a side chain capable of hydrogen bonding to a water molecule, which might then act as proton donor, also is not essential at this position. Tyr-99 is a further possible proton donor in the glutathione-binding pocket, but the Y99F mutant was essentially fully active, and the Y99FH439Q double mutant also retained ~1% of the wild-type specific activity. Thus, Tyr-99 is not acting as a surrogate proton donor/acceptor to confer activity on the H439Q mutant, and it is unlikely that the phenolic hydroxyl group plays any role in proton transfer in the wild-type enzyme. We conclude that the imidazole side chain of His-439 probably acts as proton donor/acceptor in the wild-type enzyme, leading to an improvement in the k_{cat} of approximately 100-fold compared with the H439Q and H439A mutants. The mutants may be functioning by recruiting another as yet unidentified protein side chain to act in this capacity, but it could be that the proton is simply acquired from solution, given the somewhat open structure of this part of the active site. The H439Q, H439A, and Y99FH439Q mutants all displayed a much lowered K_m for NADPH compared with the wild-type enzyme, although NADPH is bound in a separate site some distance (~18 Å) from that responsible for binding glutathione. The structural basis for this effect remains to be determined.

Glutathione reductase (EC 1.6.4.2) catalyzes the NADPH-linked reduction of oxidized glutathione:

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+$$

The product of the reaction, reduced glutathione, ensures that other thiol groups remain reduced in the cell and is particularly important in protecting the cell against oxidative stress and in the biosynthesis of DNA [for a review, see Holmgren (1985)]. The catalytic mechanism of the enzyme has been the subject of intensive investigations using a variety of techniques, chiefly protein chemical and kinetic analysis (Williams, 1976) and X-ray crystallography of the human erythrocyte enzyme (Pai & Schulz, 1983; Karplus & Schulz, 1987; Pai et al., 1988; Karplus et al., 1989). From these studies, a reaction scheme has been proposed (Pai & Schulz, 1983; Wong et al., 1988) in which reducing equivalents are passed from NADPH to oxidized glutathione via the isoalloxazine ring of enzyme-bound FAD and the redox-active

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0006-2960/89/0428-9602$01.50/0

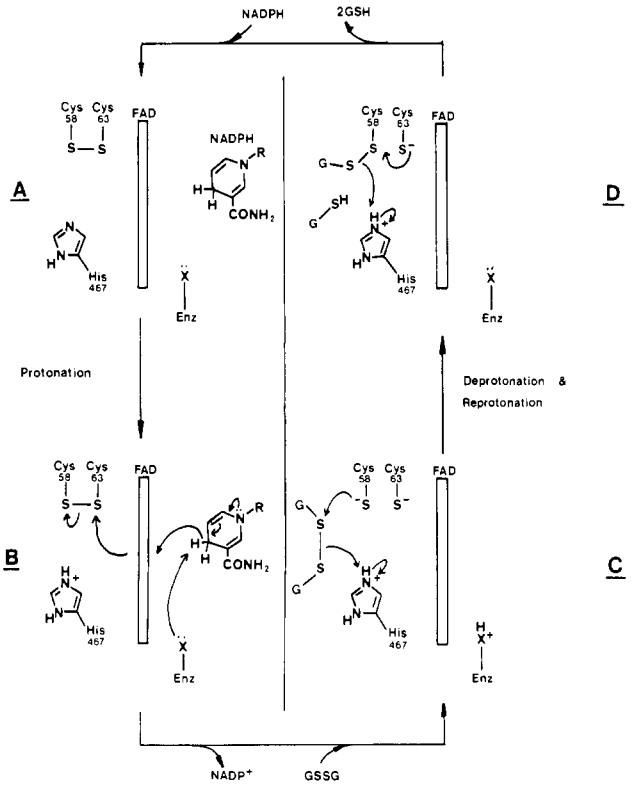

FIGURE 1: Postulated reaction mechanism for human glutathione reductase [after Pai and Schulz (1983) and Wong et al. (1988)]. The scheme, which omits some steps, should not be taken to imply that electron transfers are concerted. The base X has tentatively been identified as Lys-66 (Pai & Schulz, 1983).

disulfide bridge in each protein subunit of the dimeric enzyme (Figure 1).

The availability of a cloned (Greer & Perham, 1986) and overexpressed (Scrutton et al., 1987) gene (*gor*) encoding *Escherichia coli* glutathione reductase has already allowed us to test some aspects of the postulated mechanism by means of in vitro mutagenesis (Scrutton et al., 1988; Berry et al., 1989). Alignment of the protein sequences of the human and *E. coli* enzymes shows a high degree of primary sequence homology, indicative of a similar three-dimensional structure for the two proteins. There are, however, two notable differences between the human enzyme and its *E. coli* counterpart: the former possesses a flexible N-terminal segment (about 18 amino acid residues) of polypeptide chain and an intersubunit disulfide bridge, both of which features are absent from the *E. coli* enzyme. The successful insertion of an intersubunit disulfide bridge in the *E. coli* enzyme at a position on the 2-fold axis equivalent to that in the native human enzyme (Scrutton et al., 1988) offers compelling evidence for the close similarity in structure of the two enzymes. This lends confidence to the use of the three-dimensional structure of the human enzyme as a valid model for directed mutagenesis experiments on the *E. coli* enzyme (Berry et al., 1989).

An important feature of the catalytic mechanism (Figure 1) of the enzyme is the need for a proton donor/acceptor in the glutathione-binding site. A histidine residue has been implicated as the proton donor/acceptor in the reduction of the substrate in human glutathione reductase (Boggoram & Mannervik, 1978) and in the related enzyme dihydrolipoamide dehydrogenase (Matthews et al., 1977). X-ray crystallographic work has shown that His-467 is suitably positioned in the glutathione-binding pocket of human glutathione reductase to act in this capacity (Pai & Schulz, 1983; Karplus et al., 1989). His-439 in *E. coli* glutathione reductase is equivalent to His-467 in the human enzyme and lies within a highly conserved region of the protein sequence (Greer & Perham, 1986). Directed mutagenesis of the *E. coli* glutathione reductase has already been used to demonstrate that, although this histidine residue facilitates the catalytic activity of the enzyme, a protonatable side chain at this site is not absolutely essential to the mechanism. Thus, a small amount of residual activity was observed in a mutant enzyme in which His-439 was replaced by a glutamine residue. However, it was not resolved whether this activity was due to reliance on an alternative protonatable side chain in the enzyme, to the glutamine residue serving to hydrogen bond a water molecule that acted as an alternative proton donor/acceptor, or to some other unknown mechanism (Berry et al., 1989). We have now tackled this problem further by systematically removing the possible proton donors/acceptors in the glutathione-binding pocket. We have concentrated our efforts on residues His-439 and Tyr-99, the latter having been considered a possible, albeit unlikely, proton donor replenished by solvent (Pai & Schulz, 1983; Schirmer et al., 1989).

Materials and Methods

Materials. Complex bacteriological media were from Difco Laboratories, and all media were prepared as described in Maniatis et al. (1982). [^{35}S]dATP-αS triethylammonium salt (400 Ci/mmol) for DNA sequencing was supplied by Amersham International. Ethidium bromide, NADPH, thio-NADP$^+$, and GSSG were from the Sigma Chemical Co. Ultrapure agarose, dithiothreitol, and CsCl were from Bethesda Research Labs. Procion Red HE-7B was a gift from Dr. C. R. Lowe (Institute of Biotechnology, University of Cambridge) and was linked to CL-Sepharose 4B as described in Lowe et al. (1980). All other chemicals were of analytical grade wherever possible. Glass-distilled water was used throughout.

The restriction enzymes *Hin*dIII, *Eco*RI, *Nsi*I, *Pst*I, and *Eag*I were purchased from New England Biolaboratories. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA ligase and T4 polynucleotide kinase were from Amersham International.

E. coli strain TG1 [K12, Δ(*lac-pro*), sup E, *thi*, *hsd* D5, /F′ *tra* D36, *pro* A$^+$B$^+$, *lac* I^q, *lac* Z ΔM15] was provided by Dr. A. Gibson (MRC Laboratory of Molecular Biology, Cambridge, U.K.). *E. coli* strain SG5 [F$^-$ Δ(*his-gnd*), Δ*gor*, Δ*lac ara* D, Str^R] was previously isolated in this laboratory (Greer & Perham, 1986). *E. coli* strain BMH71-18 [K12, Δ(*lac-pro*), *sup* E, *thi*/F′ *pro* A$^+$B$^+$, *lac* I^q, *lac* Z ΔM15] was obtained from Dr. G. Winter (MRC Laboratory of Molecular Biology, Cambridge, U.K.).

Site-Directed Mutagenesis and DNA Sequencing. Site-directed mutagenesis was carried out on a derivative of M13 containing the noncoding strand of the *gor* gene (either K19gor or K19gor3′δEcoRI; see below). The mutagenic oligonucleotides 5′-TACTTCCTTTGAAAACG-3′ (Y99F), 5′-CCGTCGCCATTGCCCCAACGGCGG-3′ (H439A), and 5′-CTACAATCGCGGAATTCAACGATAAG-3′ (*Eco*RI insertion) were annealed to single-stranded template, and mutants were isolated. Mutant Y99F was constructed by using the *Eco*K/*Eco*B double primer with selection method (Carter et al., 1985; Scrutton et al., 1988), and the other mutants were constructed by using the phosphorothioate method (Taylor et al., 1985) as marketed by Amersham International. Plaques derived from the *Eco*K/*Eco*B mutagenesis reaction were screened by colony hybridization with the appropriate mutagenic oligonucleotide, and the mutations were confirmed by dideoxy sequencing (Sanger et al., 1980; Biggin et al., 1983).

Putative mutants generated by the phosphorothioate method were screened directly by dideoxy sequencing using the Sequenase system obtained from United States Biochemical Corp. The whole of the mutated gene was resequenced to ensure that no spurious mutations were introduced during the mutagenesis reactions. The mutant H439Q has already been described (Berry et al., 1989).

Plasmid Construction. Plasmid or bacteriophage RF DNA was prepared by CsCl density gradient centrifugation as described by Maniatis et al. (1982). For the purposes of screening, plasmids were prepared on a miniscale using the alkaline lysis method described in Maniatis et al. (1982). Restriction endonuclease digestion of DNA was carried out as recommended by the enzyme suppliers. The mutant genes (Y99F, H439Q, and H439A) were isolated by restricting bacteriophage RF DNA with *Eco*RI and *Hin*dIII, and the *gor* gene fragment was subcloned into the expression vector pKK223-3 restricted with the same enzymes, as described by Scrutton et al. (1987).

Growth of Cells and Purification of Glutathione Reductase. Wild-type and the mutant (H439Q, Y99F, and Y99FH439Q) glutathione reductases were purified from the *gor*-deletion strain of *E. coli* SG5 (Greer & Perham, 1986) transformed with the appropriate expression plasmid as described by Berry et al. (1989). Mutant H439A was purified from a *lac* I^q derivative of strain SG5 (strain NS3) as described below.

Measurement of Kinetic Parameters. Specific activities of wild-type and mutant glutathione reductases in the direction of glutathione reduction were measured under saturating conditions (Scrutton et al., 1987), and the kinetic parameters of the mutant enzymes were determined as described previously (Berry et al., 1989). The transhydrogenase activity of glutathione reductase was estimated at 30 °C by the thio-$NADP^+$-dependent oxidation of NADPH measured by the increase in the absorption at 398 nm. Assay mixtures contained 100 μM NADPH, 100 μM thio-$NADP^+$, the enzyme to be assayed, and 0.1 M potassium phosphate buffer, pH 7.6, to a final volume of 1 mL.

SDS–Polyacrylamide Gel Electrophoresis. Cell-free extracts of *E. coli* and samples of purified *E. coli* glutathione reductase were submitted to electrophoresis in 15% polyacrylamide slab gels in the presence of SDS and were subsequently stained with Coomassie Brilliant Blue R-250 (Scrutton et al., 1987).

Results and Discussion

Enhanced Expression of the E. coli gor Gene. We have previously described an expression system for the *E. coli gor* gene based on the plasmid pKGR, which overexpresses the protein about 200 times above the level obtained from the chromosomal *gor* gene (Scrutton et al., 1987). However, in the DNA sequence of the plasmid pKGR, there is a 700 bp stretch of DNA between the *tac* promoter and the ribosome-binding site of the *gor* gene (Figure 2); within this stretch of DNA, there is a poly(T) region of about 30 bases, originating from the Clarke and Carbon bank plasmid from which the gene was originally cloned (Greer & Perham, 1986). It was thought possible that this poly(T) region might be reducing the efficiency of transcription of the *gor* gene by acting as a termination signal for RNA polymerase. It was therefore removed as follows. A 1000 bp stretch of DNA downstream of the *gor* gene in the construct K19*gor* (Berry et al., 1989) was deleted by restricting the bacteriophage RF DNA with enzymes *Pst*I and *Nsi*I, followed by recircularization of the major fragment with DNA ligase (Figure 2). An *Eco*RI site was then engineered directly before the Shine–Delgarno sequence of the *gor* gene to yield the recombinant bacteriophage K19*gor3′δ*EcoRI. The *gor* gene could now be excised as a 1.7 kbp *Eco*RI/*Hin*dIII fragment and ligated directionally into the expression vector pKK223-3 (Figure 2).

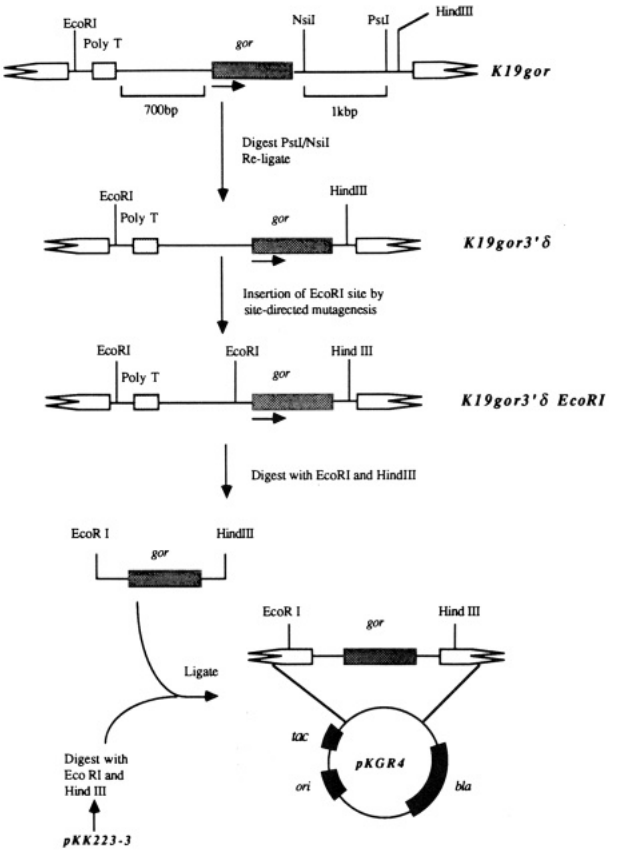

FIGURE 2: Construction of the pKGR4 expression system. The 1 kbp fragment of DNA downstream of the *gor* gene was excised from the RF DNA of bacteriophage K19gor (Berry et al., 1989) by digestion with the enzymes *Nsi*I and *Pst*I followed by religation of the major fragment to yield K19gor3′δ. An *Eco*RI cleavage site was engineered into this construct immediately upstream of the Shine–Delgarno sequence of the *gor* gene giving K19gor3′δEcoRI. The truncated *gor*-containing *Eco*RI/*Hin*dIII fragment was directionally subcloned into the expression vector pKK223-3 (to yield plasmid pKGR4), thus placing the *gor* gene under the control of the *tac* promoter.

When transformed into the *lac*I^q *E. coli* TG1 cells, the new plasmid, designated pKGR4, was found to express glutathione reductase activity at high levels, even without induction with isopropyl β-D-thiogalactopyranoside (IPTG).[1] Surprisingly, IPTG induction caused lower expression of the enzyme, suggesting that the cell may be unable to tolerate a much higher level of the enzyme than that seen in the absence of induction. The overproduction of glutathione reductase was accompanied by a marked increase in the yellow appearance of the cell-free extract, implying that flavin biosynthesis is also stimulated in cells carrying plasmid pKGR4. This could be due to sequestration of FAD by the high levels of apoenzyme generated in these cells, which may in turn result in the stimulation of the biosynthetic pathway of flavins. Nevertheless, the specific activity of glutathione reductase in the cell-free extract was no higher than that in cell-free extracts of cells expressing the *gor* gene from plasmid pKGR (Scrutton et al., 1987). SDS–polyacrylamide gel electrophoresis of samples of the cell-free extract, however, revealed that it contained large amounts of a protein with an electrophoretic mobility identical with that

[1] Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

Table I: Specific Catalytic Activities and Kinetic Parameters of the Wild-Type and Mutant Forms of *E. coli* Glutathione Reductase

enzyme	specific activities (units/mg)[a]		kinetic parameters for reduction of GSSG		
	NADPH-dependent reduction of GSSG	transhydrogenase act.	K_m(GSSG) (μM)	K_m(NADPH) (μM)	k_{cat} (min^{-1})
wild-type	252	1.2	97 ± 12	38 ± 4	36000 ± 2600
H439Q[b]	3.0	1.2	310 ± 30	<2	140 ± 10
H439A[b]	0.95	3.2	219 ± 34	<2	43 ± 4
Y99F	210	1.0	81 ± 17	53 ± 11	31600 ± 5300
Y99FH439Q[b]	1.1	0.9	66 ± 6	<2	64 ± 3

[a] Enzyme specific activities were measured at saturating concentrations of all substrates. The transhydrogenase activity was measured at 30 °C by the thio-NADP$^+$-dependent oxidation of NADPH, with both coenzymes at an initial concentration of 100 μM. [b] The true value of K_m for NADPH in this mutant could not be measured since discrimination in rate could not be achieved even at a concn. of NADPH as low as 2 μM.

of purified glutathione reductase (Figure 3). This indicates that large amounts of either apoenzyme or inactive holoenzyme were present in these cells. It was found that this could be reconstituted to form fully active enzyme during its purification by adding excess FAD to the cell-free extract before ammonium sulfate fractionation (Scrutton et al., 1987). On the basis of these reconstituted activities and the SDS–polyacrylamide gel electrophoresis (Figure 3), it was estimated that noninduced cells carrying plasmid pKGR4 were expressing glutathione reductase at levels approximately 40 000 times above that of untransformed cells.

Repeated efforts at transforming plasmid pKGR4 into the desirable *E. coli gor*-deletion strain SG5 (Greer & Perham, 1986) were unsuccessful, perhaps because strain SG5 is not *lac*I^q and the plasmid pKGR4 is therefore uncontrolled. The *lac*I^q gene was therefore introduced into strain SG5. This was achieved by first converting strain SG5 (Δ*gor*, Str^R) into a proline auxotroph (strain NS1) by means of four rounds of ampicillin enrichment (Lederberg & Zinder, 1948). An F′ episome encoding the *lac*I^q gene and the *pro*AB locus from *E. coli* strain BMH71-18 (Str^S) was then introduced by conjugation, yielding strain NS3, which was selected for by growth on minimal media containing streptomycin. Strain NS3 was shown to lack glutathione reductase activity by appropriate enzyme assay of cell-free extracts and, as expected for a bacterium carrying an F′ episome, to support growth of bacteriophage M13. The expression plasmid pKGR4 could be introduced efficiently into strain NS3 by using the $CaCl_2$ transformation procedure (Maniatis et al., 1982) and was found to be as efficiently expressed as in *E. coli* TG1 cells. In the experiments that follow, mutant enzymes derived from *gor* genes, expressed from plasmid pKGR4 in *E. coli* strain NS3 cells, were purified to homogeneity essentially as described previously (Berry et al., 1989). The only modifications to the method were that the concentration of FAD in the initial resuspension buffer (buffer A) was increased to 100 μM and that, because of the high concentration of enzyme in the starting extract, the affinity chromatography step could be dispensed with.

Properties of a Histidine-439-Alanine (H439A) Mutant. We have already demonstrated (Berry et al., 1989) that replacement of His-439 by a glutamine residue in *E. coli* glutathione reductase does not result in the total loss of enzymic activity that would be predicted if this imidazole side chain were uniquely fitted to act as the critical proton donor/acceptor required by the catalytic mechanism (Matthews et al., 1977; Pai & Schulz, 1983). The 1% residual activity observed for the mutant (H439Q) enzyme (Table I) could be due to one of several causes: to the H-bonding capacity of the glutamine residue mimicking that of a histidine residue; to the participation of another (perhaps normally superfluous) protonatable side chain in the enzyme; or to another subtle change in mechanism (Berry et al., 1989). To test the first of these possibilities, we have removed the potential for H bonding at position 439 by replacing His-439 with an alanine residue (mutant H439A).

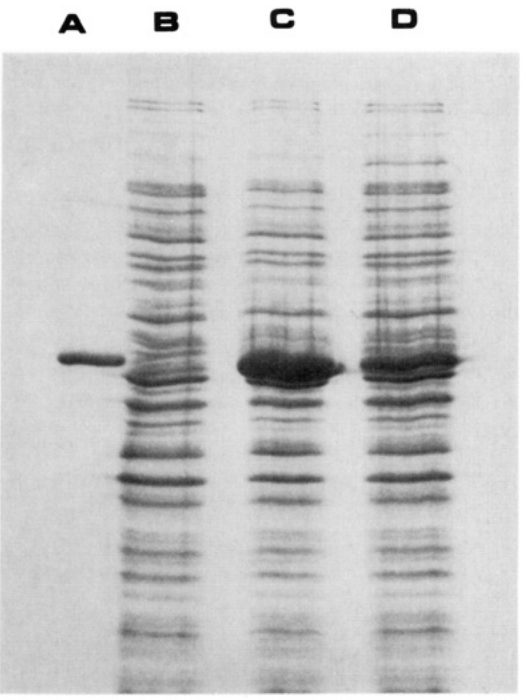

FIGURE 3: Expression of the *gor* gene in *E. coli* strain TG1. Samples of cell extracts of *E. coli* strain TG1 were prepared and analyzed by means of SDS–polyacrylamide gel electrophoresis as described under Materials and Methods. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Track A, purified wild-type glutathione reductase. Track B, cell extract of *E. coli* strain TG1. Track C, cell extract of *E. coli* strain TG1 transformed with plasmid pKGR4. Track D, cell extract of *E. coli* strain TG1 transformed with plasmid pKGR4 induced with 2 mM IPTG.

Mutant H439A was purified to homogeneity as described above and was found to possess about 0.3% of the specific activity of the wild-type enzyme (Table I), comparable with that of the H439Q mutant. This level of activity cannot be due to wild-type contamination since the enzyme was purified from a *gor* deletion strain (NS3) of *E. coli*. Kinetic analysis of mutant H439A (Table I) showed that, like mutant H439Q (Berry et al., 1989), it exhibited a much-diminished Michaelis constant for NADPH. We could not accurately determine the K_m for NADPH by simple spectrophotometric methods since the concentrations of NADPH required were too low to measure accurately. We were, however, able to measure the apparent K_m for GSSG and an apparent k_{cat} for the overall reaction at an NADPH concentration of 50 μM, finding values remarkably similar to those previously obtained for the H439Q mutant (Table I). Although the overall activity of the enzyme was severely impaired by this one mutation, the catalytic competence of the NADPH-binding site was unaltered, as

judged by the ability of the mutant enzymes (H439Q and H439A) to catalyze the NADPH/thio-NADP$^+$ transhydrogenase activity as efficiently as the wild-type enzyme (Table I).

These results reinforce our previous finding (Berry et al., 1989) that a protonatable side chain at position 439 is not absolutely essential for activity. They further demonstrate that the small residual catalytic activity in these mutants (H439A and H439Q) cannot be due to any ability of the side chain at this position to hydrogen bond to a solvent water molecule, allowing it to serve as proton donor. It must be, therefore, that the enzyme can recruit an alternative proton donor, albeit inefficiently, in order to turn over.

Properties of a Tyrosine-99-Phenylalanine (Y99F) Mutant. In human glutathione reductase, binding of GSSG is accompanied by the phenol ring of Tyr-114 moving by about 0.1 nm to lie between the two glycyl moieties of the glutathione. The tyrosine hydroxyl group is in van der Waals contact with both sulfurs of GSSG (Pai & Schulz, 1983; Karplus et al., 1989). It is possible to conceive that the phenolic hydroxyl group in this position could donate a proton to glutathione in the reaction mechanism, although, given the relatively high pK_a of unperturbed phenolic hydroxyl groups, such a proposal is possible but not probable. This tyrosine residue is conserved in the sequence of the *E. coli* enzyme (Greer & Perham, 1986) as Tyr-99, and we therefore sought to assess its contribution to the mechanism by replacing it with phenylalanine.

The Y99F mutant form of *E. coli* glutathione reductase was constructed and purified to homogeneity, as described above. Mutant Y99F had a specific activity similar to that of the wild-type enzyme (Table I), implying that residue Tyr-99 is not critically involved in proton transfer in the glutathione-binding pocket of the wild-type enzyme. Kinetic analysis of the mutant demonstrated that, like the wild-type enzyme, it followed ping-pong kinetics, and we were able to estimate the true Michaelis constants for the two substrates and the turnover number for the enzyme (Table I). The similarity of the K_m values for GSSG in the Y99F mutant and wild-type enzymes suggests that, although the phenolic hydroxyl group of Tyr-99 may be intimately associated with the bound GSSG, it does not contribute substantially to the binding of this substrate.

Despite the elimination of Tyr-99 as an essential proton donor/acceptor in the catalytic mechanism of the enzyme, it remained possible, that in the mutants H439Q and H439A, where the main proton donor/acceptor had been removed, Tyr-99 was indeed playing this part. It was essential to test this possibility, especially since an examination of the crystal structure of the human enzyme revealed few other obvious candidates for proton donor/acceptor. We therefore united the H439Q and Y99F mutations into a single gene to encode a double mutant protein and examined its catalytic activity.

Properties of the Double Mutant. The double mutant Y99FH439Q was constructed by the ligation of an *Eag*I/*Hin*dIII digest of plasmid pKGR-Y99F and an *Eag*I/*Eco*RI-cut, phosphatase-treated digest of plasmid pKGR-H439Q (Figure 4). Use of the *Hin*dIII and *Eco*RI digests minimized the possibility of reisolating either of the single mutant forms of the gene. Constructs were transformed into *E. coli* strain TG1, recombinants were selected for by growth on 2×TY ampicillin plates (Maniatis et al., 1982), and the isolation of the double mutant was verified by plasmid sequencing. The resultant construct was designated pKGR-Y99FH439Q (Figure 4). Plasmid DNA was subsequently transformed into *E. coli* strain SG5 by the Hanahan method

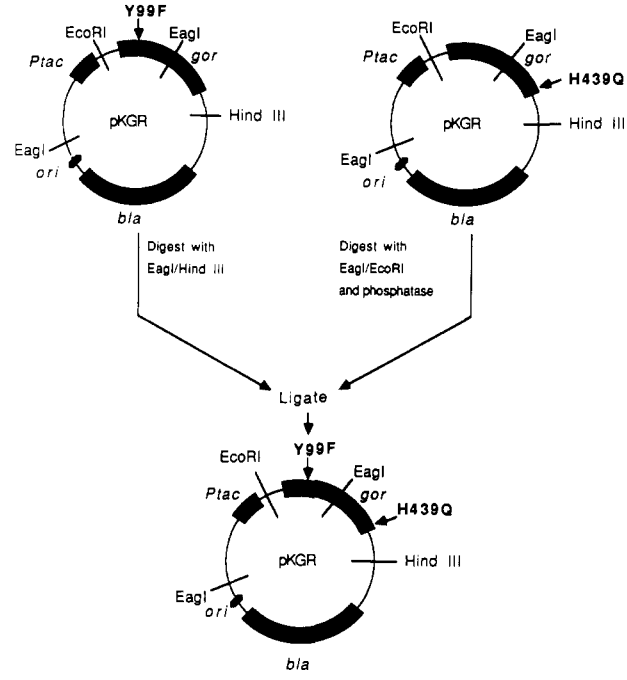

FIGURE 4: Construction of the gene encoding the mutant Y99FH439Q glutathione reductase.

(Maniatis et al., 1982) to permit expression of the mutant gene.

The Y99FH439Q double mutant protein was purified in a similar manner to the H439Q single mutant (Berry et al., 1989). Characterization of the Y99FH439Q double mutant revealed that, in its kinetics, it behaved almost identically with the H439Q single mutant. Thus, it retained about 1% of the wild-type specific catalytic activity, exhibited a much-diminished Michaelis constant for NADPH compared with wild-type enzyme, and had a transhydrogenase activity similar to that of the H439Q mutant (Table I). These results prove conclusively that Tyr-99 is not a proton donor/acceptor during the catalytic cycle of the H439Q mutant of glutathione reductase and render it unlikely that Tyr-99 plays any role in proton transfer in the wild-type enzyme.

Conclusions. Taken together with our earlier analysis of the H439Q mutant (Berry et al., 1989), our present results with the H439A mutant prove that a protonatable side chain is not essential at position 439 in the *E. coli* glutathione reductase and, further, that a side chain capable of hydrogen bonding a water molecule at that position is not essential either. It is likely, therefore, that the proton that needs to be transferred in the postulated mechanism (Figure 1) can be recruited from another source. Our present results with the Y99FH439Q mutant eliminate Tyr-99 as this alternative proton donor/acceptor, and given that the Y99F mutant is essentially unimpaired in its catalytic activity (Table I), it is unlikely that the hydroxyl group of Tyr-99 plays any significant part in proton transfer in the wild-type enzyme. It is conceivable that some as yet unidentified protein side chain acts in this capacity, but it could well be that the proton is simply acquired from the aqueous solution bathing this somewhat open part of the active site.

The advantage that accrues to the enzyme from placing a histidine residue at position 439, an approximately 100-fold increase in k_{cat} compared with the H439Q or H439A mutants (Table I), is very clear. Thus, it is probably that, in the wild-type enzyme, the imidazole side chain of His-439 does act as proton donor/acceptor in the way originally envisaged (Williams, 1976; Matthews et al., 1977; Boggoram & Mannervik, 1978; Pai & Schulz, 1983). What is of particular

interest is the way in which the enzymic mechanism alters if the glutathione-binding pocket is deprived of this particular protonatable residue. In this context, it is relevant to note that the only homologous flavoprotein disulfide oxidoreductase to lack a histidine residue at this position is mercuric reductase, in which it is replaced by a tyrosine residue (Brown et al., 1983; Misra et al., 1985; Laddaga et al., 1987). This enzyme is unique in that the substrate is not an organic disulfide.

Registry No. GSSG, 27025-41-8; NADPH, 53-57-6; His, 71-00-1; Tyr, 60-18-4; glutathione reductase, 9001-48-3.

REFERENCES

Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 1264–1269.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Boggoram, V., & Mannervik, B. (1978) *Biochem. Biophys. Res. Commun. 83*, 558–564.

Brown, N. L., Ford, S. J., Pridmore, D., & Fritzinger, D. C. (1983) *Biochemistry 22*, 4089–4095.

Carter, P., Bedouelle, M., Waye, M. M., & Winter, G. (1985) in *Oligonucleotide Site-Directed Mutagenesis in M13. A Laboratory Manual*, Anglian Biotechnology, London.

Greer, S., & Perham, R. N. (1986) *Biochemistry 25*, 2736–2742.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.

Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729.

Karplus, P. A., Pai, E. F., & Schulz, G. E. (1989) *Eur. J. Biochem. 178*, 693–703.

Laddaga, R. A., Chu, L., Misra, T. K., & Silver, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5106–5110.

Lederberg, J., & Zinder, N. (1948) *J. Am. Chem. Soc. 70*, 4267–4268.

Lowe, C. R., Hans, M., Spibey, N., & Drabble, W. T. (1980) *Anal. Biochem. 104*, 23–28.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem. 252*, 3199–3207.

Misra, T. K., Brown, N. L., Haberstroh, L., Schmidt, A., Goddette, D., & Silver, S. (1985) *Gene 34*, 253–262.

Pai, E. F., & Schulz, G. E. (1983) *J. Mol. Biol. 258*, 1751–1757.

Pai, E. F., Karplus, P. A., & Schulz, G. E. (1988) *Biochemistry 27*, 4465–4474.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. M., & Roe, B. A. (1980) *J. Mol. Biol. 143*, 161–178.

Schirmer, R. H., Krauth-Siegel, R. L., & Schulz, G. E. (1989) in *Glutathione: Chemical, Biochemical and Medical Aspects. Part A* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) pp 553–596, Wiley, New York.

Scrutton, N. S., Berry, A., & Perham, R. N. (1987) *Biochem. J. 245*, 875–880.

Scrutton, N. S., Berry, A., & Perham, R. N. (1988) *FEBS Lett. 241*, 46–50.

Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8749–8764.

Williams, C. H., Jr. (1976) *Enzymes (3rd Ed.) 13*, 89–173.

Wong, K. K., Vanoni, M. A., & Blanchard, J. S. (1988) *Biochemistry 27*, 7091–7096.

Mechanism of Interaction of *O*-Amino-D-serine with Sheep Liver Serine Hydroxymethyltransferase†

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ABSTRACT: The mechanism of interaction of *O*-amino-D-serine (OADS) with sheep liver serine hydroxymethyltransferase (EC 2.1.2.1) (SHMT) was established by measuring changes in the enzyme activity, absorption spectra, circular dichroism (CD) spectra, and stopped-flow spectrophotometry. OADS was a reversible noncompetitive inhibitor (K_i = 1.8 μM) when serine was the varied substrate. The first step in the interaction of OADS with the enzyme was the disruption of enzyme–Schiff base, characterized by the rapid disappearance of absorbance at 425 nm (6.5×10^3 M^{-1} s^{-1}) and CD intensity at 430 nm. Concomitantly, there was a rapid increase in absorbance and CD intensity at 390 nm. The spectral properties of this intermediate enabled its identification as pyridoxal 5′-phosphate (PLP). These changes were followed by a slow unimolecular step (2×10^{-3} s^{-1}) leading to the formation of PLP–OADS oxime, which was confirmed by its absorbance and fluorescence spectra and retention time on high-performance liquid chromatography. The PLP–OADS oxime was displaced from the enzyme by the addition of PLP as evidenced by the restoration of complete enzyme activity as well as by the spectral properties. The unique feature of the mechanism proposed for the interaction of OADS with sheep liver SHMT was the formation of PLP as an intermediate.

Serine hydroxymethyltransferase [L-serine:tetrahydrofolate; 5,10-hydroxymethyltransferase (SHMT), EC 2.1.2.1] catalyzes the conversion of L-serine to glycine and tetrahydrofolate (H_4folate)[1] to 5,10-methylene-H_4folate. The role of SHMT in cellular proliferation was reviewed (Schirch, 1982; Appaji Rao et al., 1987). D-Cycloserine (DCS) (Figure 1) functioned

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; H_4folate, 5,6,7,8-tetrahydrofolate; DCS, D-cycloserine; OADS, *O*-amino-D-serine; EDTA, ethylenediaminetetraacetic acid disodium salt; 2-ME, 2-mercaptoethanol; CM, carboxymethyl; A_{280}, absorbance at 280 nm; A_{425}, absorbance at 425 nm; CD, circular dichroism; TFA, trifluoroacetic acid.

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